

Restriction Fragment Length Polymorphism Analysis Using IS6110 as an Epidemiological Marker in Tuberculosis

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The mycobacterial insertion sequence IS6110 has been shown to be present in multiple copies in the chromosome of *Mycobacterium tuberculosis*. IS6110 restriction fragment length polymorphism analysis of strains isolated from patients who developed tuberculosis showed identical patterns over a 2- to 3-year period. In contrast, a high degree of polymorphism was observed between strains of the *M. tuberculosis* complex isolated from different patients. This study demonstrates that the presence of IS6110 does not induce in vivo major genomic rearrangements over a 2- to 3-year period and confirms its use as a valuable epidemiological marker in tuberculosis.

Tuberculosis is a mycobacterial infection that continues to be an important cause of human morbidity and mortality worldwide. Conventional diagnosis of tuberculosis depends on isolation, identification, and biochemical testing of the pathogenic bacteria with procedures usually requiring 4 to 8 weeks. Epidemiological studies with techniques which allow differentiation of strains within the *Mycobacterium tuberculosis* group are important for limiting the dissemination of the disease. Phage typing has been used to differentiate between *M. tuberculosis* strains, but since only a small number of phage types are recognized, its value in epidemiological studies of tuberculosis is limited (3, 10). More sophisticated techniques employing restriction fragment length polymorphism (RFLP) analysis have detected genotypic variations among members of the *M. tuberculosis* and *Mycobacterium avium* complex (4, 9) and showed high genomic conservation in *M. avium* subsp. *paratuberculosis* and *Mycobacterium leprae* strains (1, 2, 5). These studies showed that RFLP analysis could be used to obtain "fingerprints" for different isolates of *M. tuberculosis*.

Two insertion sequences of the IS3 family, IS6110 (12, 13) and IS986 (8), have been identified in members of the *M. tuberculosis* complex; IS986 differs from IS6110 by three nucleotides. Such allelic variations have previously been described for the well-defined insertion sequence IS7 (11). We name these variants iso-IS6110 elements. Four to 20 copies of iso-IS6110 have been found scattered throughout the genome of *M. tuberculosis*, while strains of *M. bovis* contain a single copy (12). An analysis of the distribution of iso-IS6110 in *M. tuberculosis* strains isolated from different patients revealed different genomic arrangements of the sequences. However, when this insertion sequence was used as a probe to analyze the RFLP patterns of strains isolated from patients of the same tuberculosis outbreak, identical patterns were observed (6). In addition, no polymorphism was detected in experimental infection of guinea pigs with *M. tuberculosis* strains over a period of 3 months. These

studies suggest that this sequence could be useful in epidemiological studies (6).

To test the long-term reliability of this RFLP, we have analyzed RFLP patterns in *M. tuberculosis* strains from patients who presented relapsed tuberculosis after a period of 2 or 3 years. Ten *M. tuberculosis* strains, isolated from five patients, were analyzed (Table 1).

DNA from *M. tuberculosis* strains was prepared as follows: 10-ml cultures in 7H9 Middlebrook medium supplemented with ADC (Difco) were grown at 37°C for 3 weeks, and then cycloserine (1 mg/ml) was added for 24 h. The cells were centrifuged and resuspended in 0.25 ml of 25% sucrose–50 mM Tris buffer (pH 8.0)–50 mM EDTA–500 µg of lysozyme ml⁻¹ and incubated for 1 h at 37°C. Tris buffer (100 mM) (pH 8.0)–400 µg of proteinase K ml⁻¹–1% sodium dodecyl sulfate (0.25 ml, total) was then added, the mixture was incubated at 55°C for 2 h, and DNA was extracted with phenol-chloroform and ethanol. Total DNA (5 µg) was digested with *Pst*I, electrophoresed on an agarose gel, and transferred to Hybond-N membrane filters as described previously (7).

DNA blots were hybridized by using IS6110 as probe. The entire IS6110 was isolated from *M. tuberculosis* H37rv DNA

TABLE 1. Strains used in this study^a

Patient	Origin	Strain no. (isolation yr)	Source	Lane in Fig. 1
1	Guadeloupe	861484 (1986)	Sputum	3
		891584 (1989)	Sputum	4
2	Guadeloupe	860962 (1986)	Sputum	5
		880471 (1988)	Sputum	6
3	Paris, France	870589 (1987)	Sputum	7
		890003 (1989)	Sputum	8
4	Loiret, France	870092 (1989)	Sputum	9
		891882 (1989)	Gastric washing	10
5	Guadeloupe	861482 (1986)	Sputum	11
		880954 (1988)	Sputum	12

^a Strains were susceptible to all antituberculous drugs except those isolated from patient 3 which were resistant to isoniazid, rifampin, ethambutol, and streptomycin.

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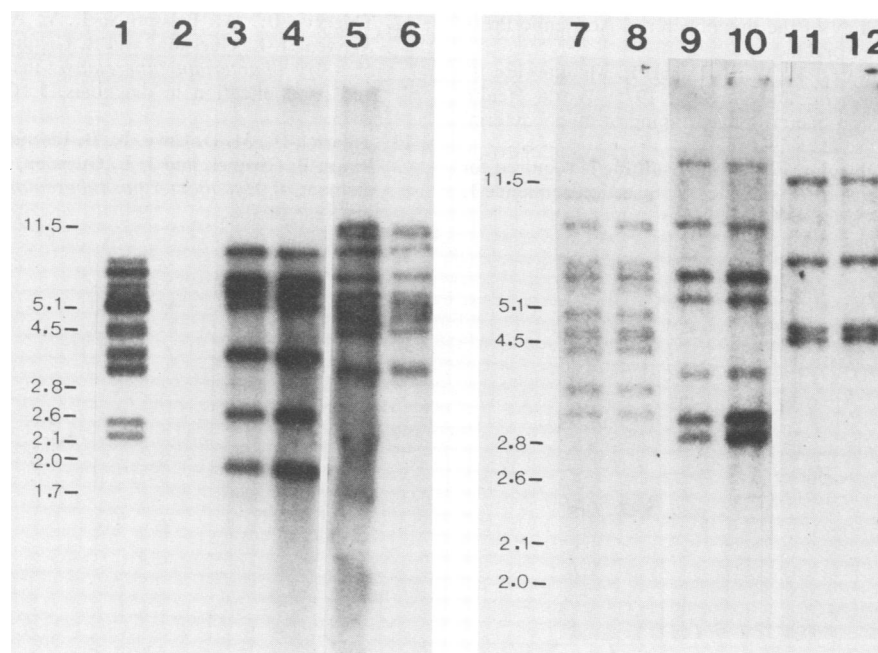


FIG. 1. Southern hybridization analysis of *M. tuberculosis* strains by using [32 P]IS6110 DNA as probe. Lanes: 1, *M. tuberculosis* H37Rv; 2, *M. smegmatis* mc²155; 3 to 12, *M. tuberculosis* 861484 (lane 3), 891584 (lane 4), 860962 (lane 5), 8800471 (lane 6), 870589 (lane 7), 890003 (lane 8), 870092 (lane 9), 8901882 (lane 10), 861482 (lane 11), and 880954 (lane 12).

(polymerase chain reaction) by using the following primer oligonucleotides: (i) GGG AAT TCG ACG GAC GTC GTG ACC AGA AGT C and (ii) GGG AAT TCG TGT ACA AAA TGT GGA CAA GTA, the former starting 51 nucleotides upstream and the latter starting 84 nucleotides downstream of the published sequence. Both oligomers contained *Eco*RI sites. A 1.4-kb amplified fragment was digested with *Eco*RI and cloned into the *Eco*RI site of pUC18 to provide pMT03. The *Eco*RI fragment was isolated by standard procedures (7) and used as a probe.

To detect RFLPs, DNA from different *M. tuberculosis* strains was digested with restriction enzyme *Pst*I; there is no site present in the IS element. Southern blot hybridizations of DNA from the *M. tuberculosis* strains are shown in Fig. 1. A high degree of polymorphism of IS6110-containing fragments was observed in the *M. tuberculosis* strains isolated from different patients, whereas identical patterns were observed in strains isolated from the same patient. In each case, patterns of strains before and after the relapse were identical, suggesting that IS6110 does not induce major chromosomal rearrangements during a period of latency of *M. tuberculosis* strains in humans. This also shows that relapse was not the result of a new infection in these patients.

RFLP studies are used to discriminate between individuals by highlighting minor chromosomal changes that are not always related to a variation in the phenotype. The stability of the polymorphism detected with IS6110-derived probes allows this method to be used for the identification of clonal populations, a prerequisite for studies of transmission of tuberculosis. In addition, RFLP analysis of *M. tuberculosis* strains should distinguish between reactivation of ancient lesions and new infections. This will be of value when examining associations with other characteristics such as levels of virulence or the origin of antibiotic-resistant strains of *M. tuberculosis*.

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REFERENCES

1. Clark-Curtiss, J. E. 1990. Genome structure of mycobacteria, p. 77-96. In J. McFadden (ed.), *Molecular biology of the mycobacteria*. Academic Press, Inc. (London), Ltd., London.
2. Clark-Curtiss, J. E., and P. W. Gerald. 1989. Conservation of genomic sequences among isolates of *Mycobacterium leprae*. *J. Bacteriol.* 171:4844-4851.
3. Clavel-Sérés, S., and F. Clément. 1984. Répartition des lyso-types de *Mycobacterium tuberculosis* en relation avec le pays d'origine du malade. *Ann. Inst. Pasteur Microbiol.* 135B:35-44.
4. Eisenach, K., J. T. Crawford, and J. H. Bates. 1988. Repetitive DNA sequences as probes for *Mycobacterium tuberculosis*. *J. Clin. Microbiol.* 26:2240-2245.
5. Green, E. P., M. L. V. Tizard, M. T. Moss, J. Thompson, D. J. Winterbourne, J. L. MacFadden, and J. Hermon-Taylor. 1989. Sequence and characteristics of IS900, an insertion element identified in a human Crohn's disease of *M. paratuberculosis*. *Nucleic Acids Res.* 17:9063-9073.
6. Hermans, P. W. M., D. V. van Soolingen, D. J. W., A. R. J. Schuitema, R. A. McAdam, D. Catty, and J. D. A. van Embden. 1990. Insertion element IS986 from *Mycobacterium tuberculosis*: a useful tool for diagnosis and epidemiology of tuberculosis. *J. Clin. Microbiol.* 28:2051-2058.
7. Martin, C., J. Timm, J. Raubier, R. Gomez-Lus, J. Davies, and B. Gicquel. 1990. Transposition of an antibiotic resistance element in mycobacteria. *Nature (London)* 345:739-743.
8. McAdam, R. A., P. W. M. Hermans, D. Van Soolingen, Z. F. Zainuddin, D. Catty, J. D. A. van Embden, and J. W. Dale. 1990. Characterization of a *Mycobacterium tuberculosis* insertion sequence belonging to the IS3 family. *Mol. Microbiol.* 4:1607-1613.
9. McFadden, J., Z. Kunze, and P. Seechurn. 1990. DNA probes for detection and identification, p. 139-172. In J. McFadden

- (ed.), Molecular biology of the mycobacteria. Academic Press Inc. (London), Ltd., London.
10. **Rado, T. A., J. H. Bates, H. W. B. Engel, E. Mankiewicz, T. Murohashi, Y. Mizuguchi, and L. Sula.** 1975. World Health Organization studies on bacteriophage typing of mycobacteria. *Am. Rev. Respir. Dis.* **111**:459-468.
 11. **Sekine, Y., and E. Ohtsubo.** 1989. Frameshifting is required for production of the transposase encoded by insertion sequence 1. *Proc. Natl. Acad. Sci. USA* **86**:4609-4613.
 12. **Thierry, D., A. Brisson-Nôel, V. Vincent-Lévy-Frébault, S. Nguyen, J.-L. Guesdon, and B. Gicquel.** 1990. Characterization of a *Mycobacterium tuberculosis* insertion sequence, IS6110, and its application in diagnosis. *J. Clin. Microbiol.* **28**:2668-2673.
 13. **Thierry, D., M. D. Cave, K. D. Eisenach, J. T. Crawford, J. H. Bates, B. Gicquel, and J. L. Guesdon.** 1990. IS6110, and IS-like element of *Mycobacterium tuberculosis* complex. *Nucleic Acids Res.* **18**:188.